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Synthesis and Biological Activity of Nitro-Substituted Cyclopenta-Fused PAH

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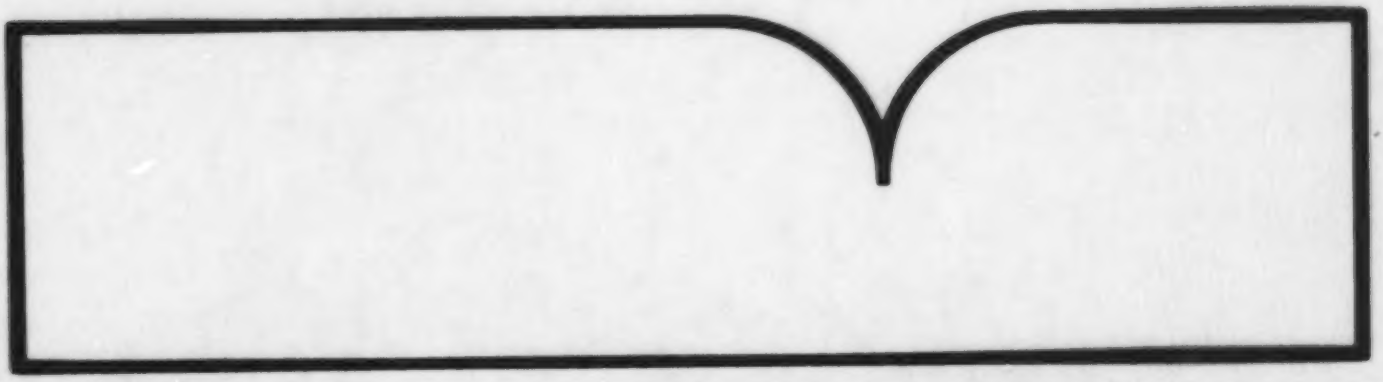

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NITRO-SUBSTITUTED CYCLOPENTA-FUSED PAH

by

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16. ABSTRACT PAH containing a peripherally fused cyclopenta ring are genotoxically active in Salmonella and mammalian cells and have been identified in combustion emissions. Since the cyclopenta ring is predicted to be susceptible to electrophilic attack, nitrosubsitituted cyclopenta-fused PAH may be formed in the presence of NO^x, particularly in diesel exhaust, and hence be present in the environment. Nitro derivatives of cyclopenta (cd) pyrene and the cyclopenta-fused benzanthracene isomers were prepared by reaction of the PAH with dinitrogen tetroxide. The purifed products were characterized by mass spectroscopy, ^1H NMR, UV-vis and HPLC. In all cases, nitro substitution occurred on the cyclopenta ring in accord with expectation. Preliminary assay in S. typhimurium indicates that 4-nitro-cyclopenta(cd)pyrene is mutagenically active without exogenous metabolic activation in nitroreductase-competent strains. The high resonance stability of the nitrenium ion formed from the putative hydroxylamine intermediate may be correlated with the mutagenicity of these compounds. The results of this study have allowed us to test this hypothesis and will aid in development of structure-activity relationships of nitroPAH.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NITRO-SUBSTITUTED CYCLOPENTA-FUSED PAH

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INTRODUCTION

Nitro-substituted polycyclic aromatic hydrocarbons have recently stimulated considerable interest because of their high mutagenic activity in the Ames assay and prevalence in the environment. They were first detected in photocopier toners (1) and have since been identified in coal fly ash particles (2), cigarette smoke condensates (3), emissions from diesel engines (4) and ambient air particulate matter (5).

Relatively little was known about the mechanism(s) by which these compounds exert their mutagenic activity. Rosenkranz and Mermelstein (6) recently initiated a series of computer-assisted studies aimed at identifying particular structural features associated with the mutagenic activity of nitroarenes. Klopman and Rosenkranz (7) concluded that nitroarenes require activation by the target cells before becoming biologically active; so-called "direct-acting" nitroaromatics are presumably converted by a family of nitroreductases to arylhydroxylamines that are mutagenic themselves or are conjugated to form hydroxamic acid esters that are non-enzymatically converted to arylnitrenium ions.

The present study attempts to extend the assessment of structure-activity relationships among the nitroarenes by synthesis and bioassay of nitro derivatives of benz(a)aceanthrylene and cyclopenta(cd)pyrene (Fig. 1). We are interested in compounds substituted on the etheno bridge of the cyclopenta ring as the most likely products of atmo-

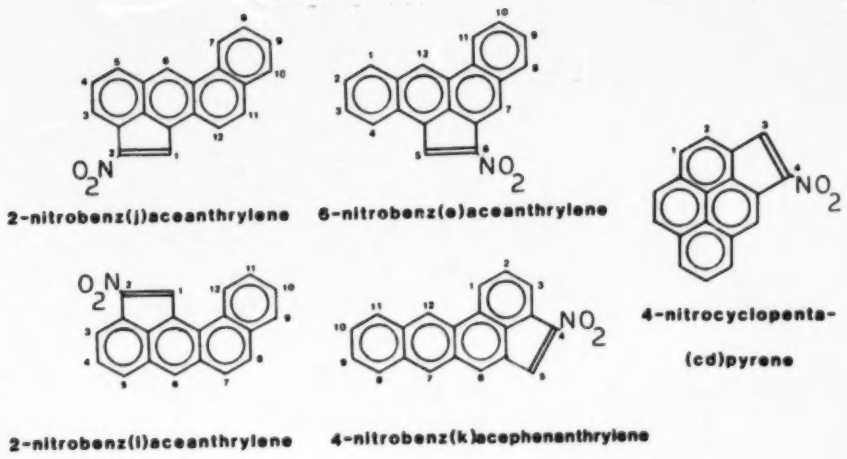


FIGURE 1. Nitrated Cyclopenta-Fused Isomers of Benz(a)anthracene and Cyclopenta(cd)pyrene.

spheric nitration of the parent PAH. By synthesis and testing of a series of compounds in the same laboratory, the problem of interlaboratory variability can be avoided. The assay results for each compound can thus be directly compared for the series, and may also be compared with those of the parent hydrocarbons. This study will provide an opportunity to test several hypotheses concerning structural requirements for the activation of the nitroarenes.

We report here the preliminary findings for two compounds: 2-nitrobenz(1)aceanthrylene (2-nitroB(1)A) and 4-nitrocyclopenta(cd)pyrene (4-nitroCPP). The arylnitrenium ions which would be formed are expected to be highly stabilized, as illustrated in Fig. 2 for 2-NitroB(1)A. The positive charge developed on the nitrogen atom is an allylic extension of the resonance-stabilized benzylic carbonium ion fused to the meso carbon of the benz(a)anthracene substructure. The accessibility of the nitro group to enzymatic reduction is crucial to activation by the proposed route and hence both steric requirements for nitro reduction and the role of resonance stabilization of the putative nitrenium ion intermediate can be examined.

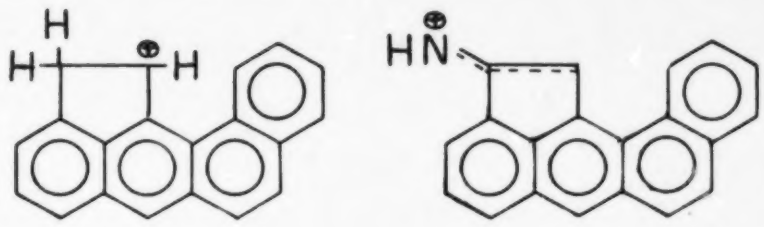


FIGURE 2. Formation of Arylnitrenium Ion in 2-Nitrobenz(1)aceanthrylene.

MATERIALS AND METHODS

Synthesis. The parent hydrocarbons were synthesized by published methods (8,9). The hydrocarbons were then nitrated by the method of Radner (10); 0.5 mL of N_2O_4, poured from a cooled tank (Matheson), was added to 20 mL of CH_2Cl_2. A stoichiometric amount of this solution was added to the parent hydrocarbon (dissolved in CH_2Cl_2; 5 mg/mL). The course of the reaction was monitored by UV-vis spectrophotometry; when the spectrum of the starting hydrocarbon disappeared, the reaction was terminated by evaporating the CH_2Cl_2 under a stream of nitrogen.

The crude mixture was then applied to a column of silica gel (Kieselgel 60, 230-400 mesh ASTM, EM Reagents) and chromatographed with CCl_4. The residual parent hydrocarbon eluted first, as a yellow band, followed by a deep red band of nitrated product. The nitro compounds were collected, dried under a stream of N_2, then recrystallized from acetone (melting point: 2-nitroB(1)A, 245°; 4-nitroCPP, 183°).

Bacterial Mutagenicity Assays. Bacterial mutagenicity assays were performed at the Health Effects Research Laboratory of the Environmental Protection Agency, Research Triangle Park, NC, according to the procedure of Ames et al. (11). All strains of **Salmonella typhimirium** and nutrient broths were prepared as described in the EPA standard protocol (12). True revertance to prototrophy was confirmed by plating small colonies onto histidine-free media and, in all cases, growth was observed.

Since HPLC analysis of DMSO solutions demonstrated that the nitro compounds are unstable in DMSO, the standard procedure was varied by dissolving the test compounds in 50 L of acetone. Spontaneous revertance was measured by addition of the same amount of acetone to a plate.

Instrumentation. ^{1}H NMR spectra were obtained on a Bruker WM250 spectrometer in acetone D_6. Signals are in ppm downfield from TMS. Mass spectrometry was performed on a VG Micromass 7070F mass spectrometer operated in the electron impact mode at 70eV. UV-vis spectra were obtained on a Cary model 15 spectrophotometer.

RESULTS

Structural Assignments. 2-nitroB(1)A. The ^{1}H NMR spectrum (Fig. 3A) shows the strongly deshielded bay region proton as a doublet at 9.1 ppm. This signal overlaps the singlet resonance from the proton at C6. The doublet from the proton on C5, which is adjacent to the strongly electron-withdrawing nitro group, appears at 8.9 ppm. The signal from the etheno-bridge proton, deshielded by the neighboring nitro group and the ring current within the "pseudo fjord" at 8.58 ppm, confirms the location of the nitro group on the etheno bridge. The mass spectrum (Fig. 3B) shows the expected M^+ peak at m/z=297, with major fragments at m/z=267 (M^+-NO) and m/z=250 (M^+-HNO_2). The UV-vis spectrum shows the broadened peaks characteristic of nitroarenes (Fig. 3C).

4 and 5-Nitrobenz(k)acephenanthrylene Mixture. The mass spectrum (Fig. 4A) demonstrates the presence of mononitrated benz(k)acephenanthrylene, with ions at m/z=297 (M^+), m/z=267 (M^+-NO) and m/z=250 (M^+-HNO_2). The ^{1}H NMR spectrum (Fig. 4B) suggests a mixture of two compounds, nitrated at either position on the cyclopenta ring. The presence of two compounds is confirmed by thin-layer chromatography (the appearance of two distinct spots on silica, solvent benzene/hexane). Preparative separation of the two products will be attempted by thick-layer chromatography on silica.

4-nitroCPP. The singlet for the proton of C5, which is in close proximity to the nitro group, appears at 9.2 ppm and is the most deshielded signal in the ^{1}H NMR spectrum (Fig. 5A). A singlet at 8.55 ppm is assigned to the proton on the etheno bridge and is also deshielded relative to the bridge proton resonances of the parent CPP. The mass spectrum (Fig.

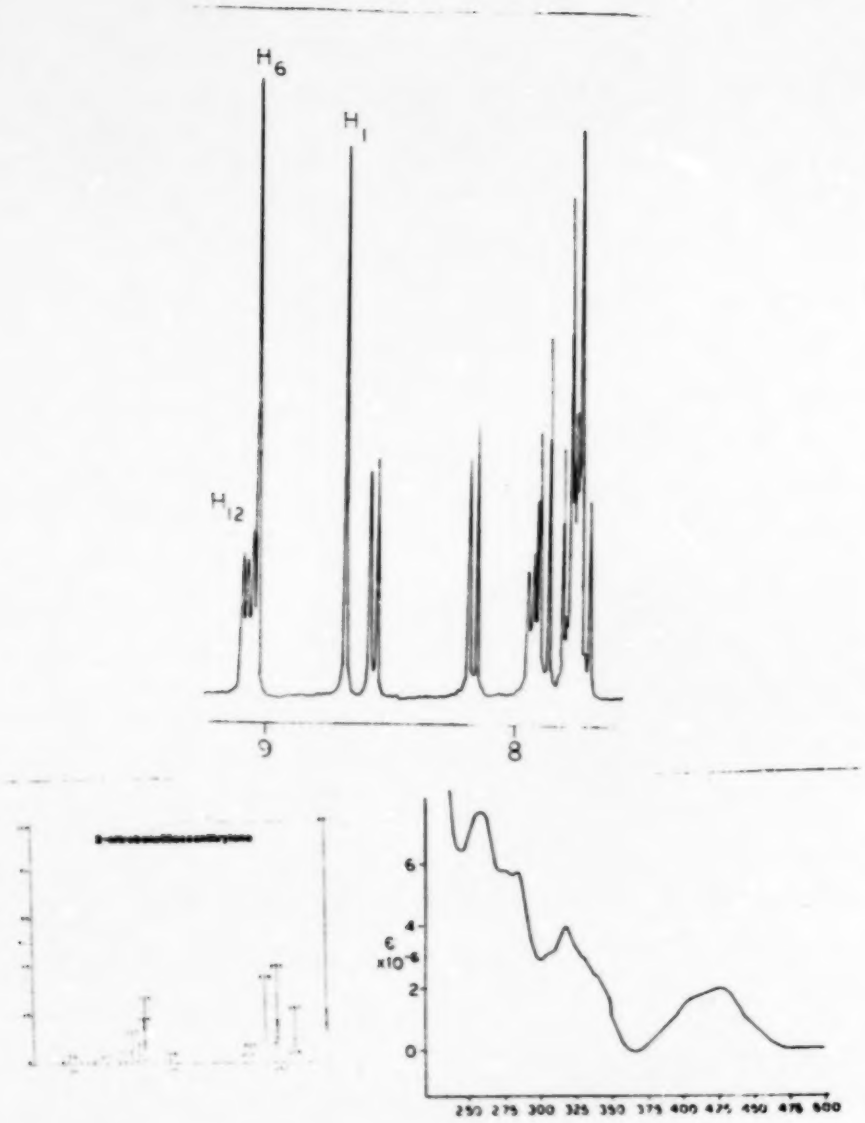


FIGURE 3. Spectra of 2-nitrobenz(l)aceanthrylene.
a. ^{1}H NMR b. Mass spectrum c. UV-vis

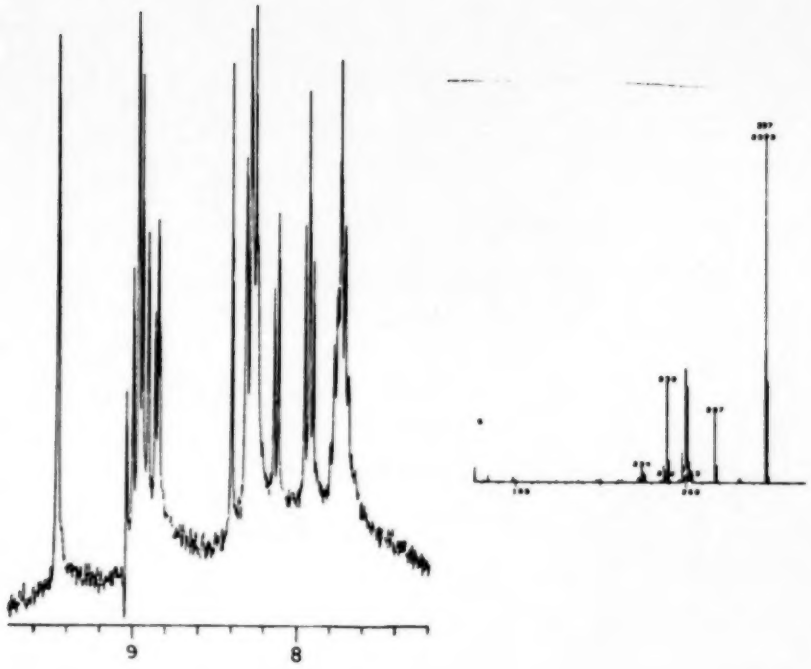
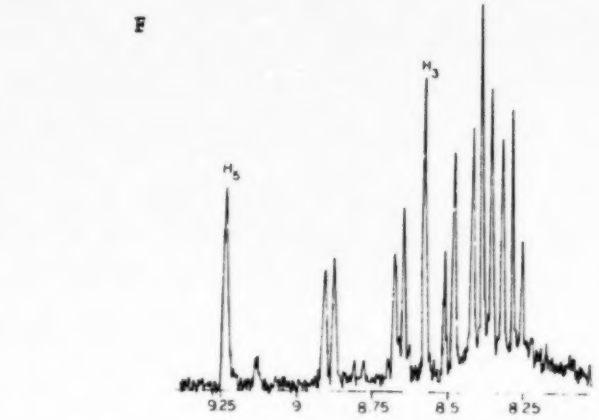


FIGURE 4. Spectra of 4 and 5 nitrobenz(k)aceanthrylene.

5B) shows the molecular ion at m/z=271 and major fragments at m/z=241 (M^+-NO) and m/z=224 (M^+-HNO_2). The UV-vis spectrum is shown in Fig. 5C.

Bacterial Mutagenicity Assays. The bacterial mutagenicity data are summarized in Fig. 6.

In TA98, which detects frameshift mutations (12) and contains an "r-factor" plasmid that inactivates the DNA repair system, thus enhancing the detectability of mutations, 4-nitroCPP shows substantially more activity than 2-nitroB(1)A (Fig. 6A). Nevertheless, the activity of 4-



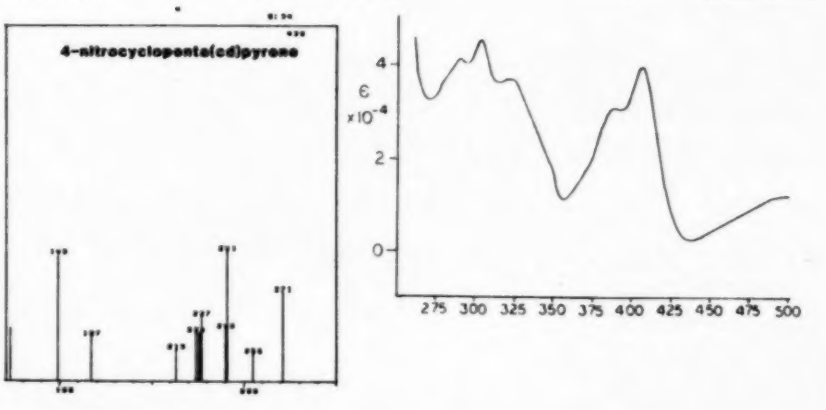


FIGURE 5. Spectra of 4-nitrocyclopenta(cd)pyrene.
a. ^{1}H NMR b. Mass Spectrum c. UV-Vis

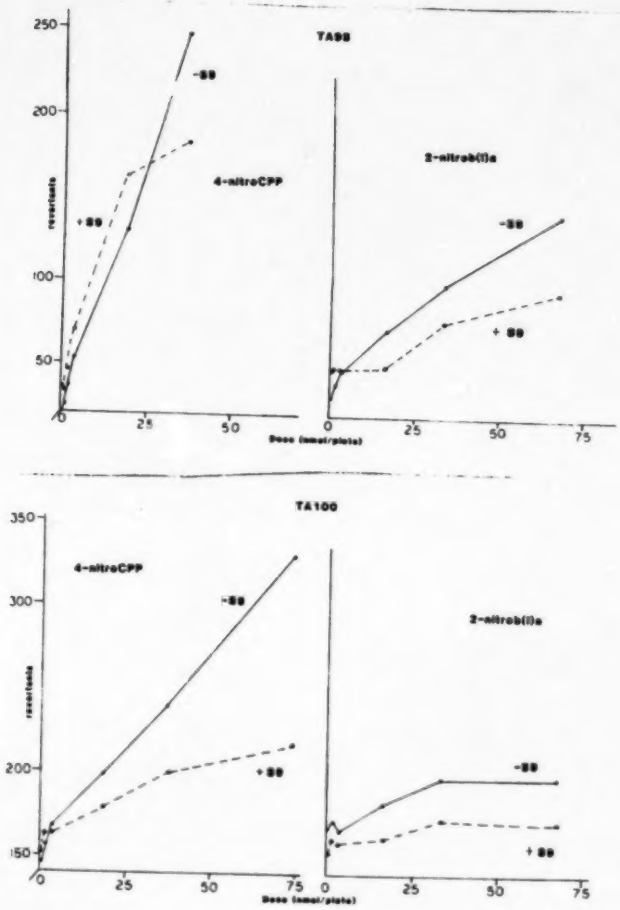


FIGURE 6. Bacterial mutagenicity assays in S. typhimurium for 4-nitrocyclopentapyrene and 2-nitrobenz(l)aceanthrylene. The results represent an average of two experiments performed on separate occasions with each dose repeated in triplicate. a. TA98 b. TA100 c. TA1535 d. TA1537 e. TA1538

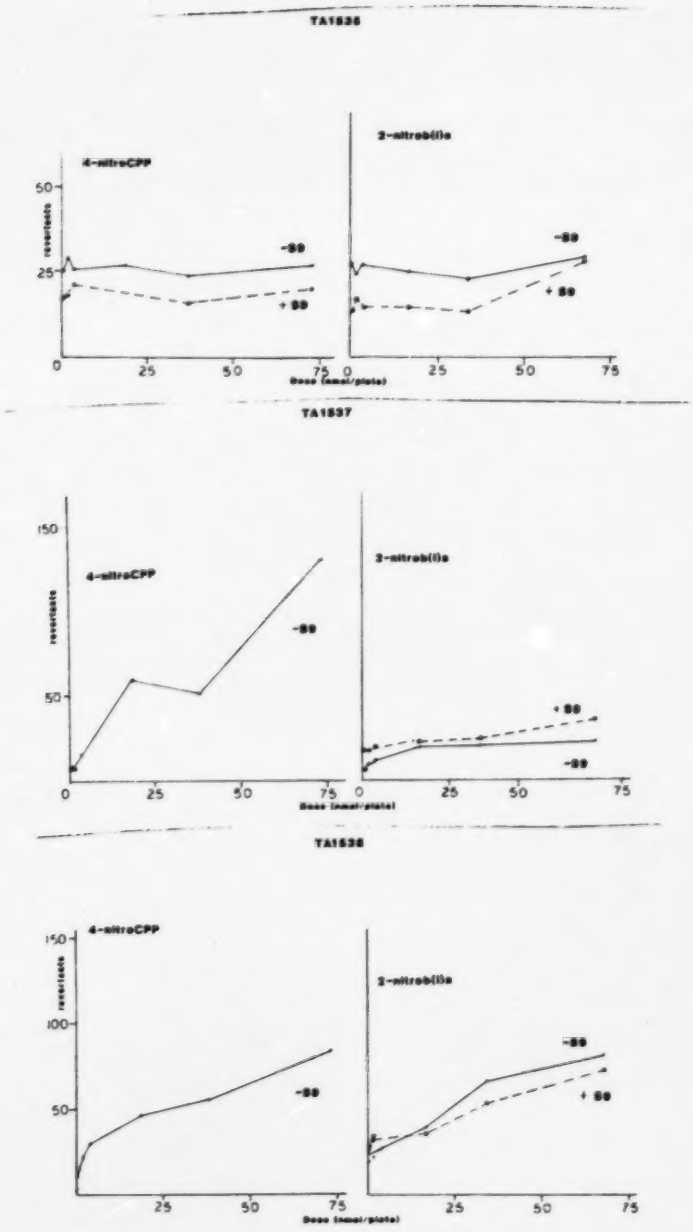


FIGURE 6. Bacterial mutagenicity assays in S. Typhimurium .... (continued)

nitroCPP in this strain (5.9 rev/nmol-S9) is low in comparison to the more potent known "direct-acting" mutagens; for example, in this laboratory, 1-nitropyrene produced approximately 250-300 rev/nmol in TA98 in the absence of S9 (13). Interestingly, 4-nitroCPP shows no diminution of activity in the presence of S9 (6.9 rev/nmol). 2-nitroB(1)A shows a slight decrease in activity (1.65 rev/nmol+S9, 0.7 rev/nmol-S9). Either with or without activation, both 4-nitroCPP and 2-nitroB(1)A are less active in TA98 than the parent hydrocarbons (80 rev/nmol and 20 rev/nmol+S9) (14). At the highest dose of both compounds given to TA98 (75 nmol/plate), toxicity was evident.

The response of strain TA100, which detects base-pair substitution and also contains the r-factor plasmid, is shown for the two nitro PAH in Figure 6B. Consistent with other direct-acting nitroarenes, both compounds produce much lower activity in TA100 than in TA98. Strain TA1535 (Fig. 6C), which also detects base-pair substitutions but lacks the plasmid for repair deficiency, shows no activity towards either nitroPAH. However, strain TA1537 (Fig. 6D) shows moderate activity and TA1538 (Fig. 6E) behaves in a similar manner. The results in TA1537 and TA1538 are consistent with the activity in TA98, since all three strains are sensitive to frameshift mutations with the former two lacking the plasmid.

DISCUSSION

In dry CH_2Cl_2, the reaction of PAH and nitrogen dioxide is presumed to involve electrophilic attack of the NO_2 radical followed by oxidation of the intermediate aryl radical to yield the nitroPAH (Fig. 7)(15).

Since the cyclopenta ring contains a highly localized double bond and is a region of high electron density, it should be susceptible to electrophilic attack; hence, the reaction leading to nitration selectively occurs at the cyclopenta ring. For B(1)A and CPP, the charge distribution on the etheno bridge forming the cyclopenta ring is highly asymmetric so that only a single product is obtained and purification is straightforward. In the case of benz(k)acephenanthrylene, however, the electron density is nearly symmetric and nitration occurs at both carbons of the etheno bridge.

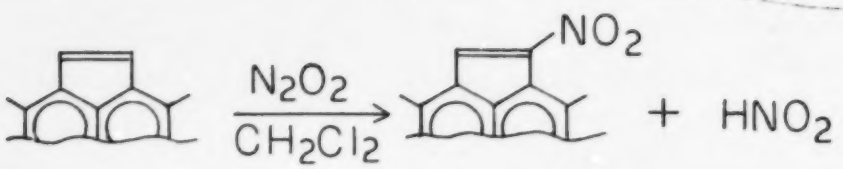


FIGURE 7. Proposed reaction mechanism of formation of nitro cyclopenta-fused PAH

Biological Activity. The results of the Ames assay indicate both 4-nitroCPP and 2-nitroB(1)A are biologically active. Consistent with other nitroarenes previously tested, they do not require S9 activation, suggesting that they are "direct-acting" mutagens; in addition, they appear to induce primarily frameshift mutations.

However, these compounds are significantly less active than other nitroarenes of similar molecular weight, e.g., 1-nitropyrene and 3-nitrofluoranthene, nor are they nearly as active as the parent compounds (which require S9 activation). Moreover, preliminary results on 2-nitroB(1)A in the mammalian C3H10T1/2 morphological cell transformation assay indicate that this compound is highly toxic but induces little or no transformation (16). Possible explanations for this relative lack of activity are:

(i) These compounds may be poor substrates for the nitroreductase enzymes; little is known of the geometry of the active site. 2-nitroB(1)A is even less active than CPP perhaps as a result of the different stereochemical environments of the nitro group. In 2-nitroB(1)A, the nitro group is located at one end of a helical molecule and its access to the active site of the reductase enzymes or of the putative nitrenium ion to reactive sites on DNA may therefore be hindered. The nitro group of CPP is on

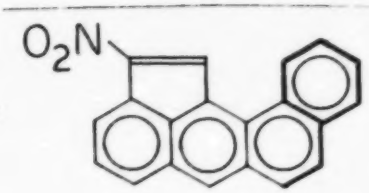


FIGURE 8. Formula and significant fragment of 2-nitroB(1)A. Heavy lines indicate deactivating fragments (7).

the periphery of a planar molecule which does not appear to impose any such steric constraints.

(ii) Klopman and Rosenkranz (7) have devised a system for predicting activities of nitroPAH and, according to this system, neither compound should be active. They categorize all the nitroarenes tested as active (greater than 1 rev/-nmol) or inactive and identified molecular fragments commont to each group. Analysis of the structure of 2-nitroB(1)A according to this correlation scheme reveals a deactivating fragment (Fig. 8), which would predict a lack of mutagenicity. 4-nitroCPP possesses neither activating or deactivating fragments, a situation also considered to be predictive of little or no direct-acting mutagenicity. Although the behavior of 2-nitroB(1)A and 4-nitroCPP conforms to the predictions of this empirical structure-activity correlation, biochemical explanations for these predictions are completely lacking.

(iii) Bacterial nitroreductase enzymes may not be involved at all in the activation of nitro-substituted cyclopenta-fused compounds. This hypothesis is suggested by the case of 5-nitroacenaphthene (17), which is a very active mutagen and entirely S9-dependent. Perhaps the nitro group alters the electronic structure of the molecule so an as-yet unidentified active metabolite can be formed. Or maybe the nitro group must be removed prior to activation of these compounds; this step may in fact be rate-limiting.

Ames testing of the remainder of the compounds in the nitrobenzaceanthrylene series will reveal whether low activity is characteristic of this class of compounds or if one or more show greater activity. X-ray crystallography will also be performed on as many in the series as possible to determine their exact three-dimensional structure, and therefore evaluate if steric or electronic factors play a role.

Metabolism studies will also enable us to determine if the nitro group on the etheno bridge is reduced less readily than a nitro group on an aromatic ring. How the nitro group affects S9 activity also must be determined. Such studies in **S. typhimurium** will help elucidate whether and to what extent nitroreduction is hindered by structural constraints with the eventual goal of determining the steric requirements of nitroreductase active sites.

ACKNOWLEDGEMENTS

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